Quantification of Lepidoptera wing patterns using an image analyzer.

Jack J. Windig

Section of Evolutionary Biology, Department of Population Biology, University of Leiden, Schelpenkade 14A, 2313 ZT Leiden, The Netherlands.

Abstract. The use of an image analyser for measuring butterfly wings is discussed. As an example the measurement of the phenotypic plasticity in the wing pattern of a tropical butterfly is described in detail. A set of 7 characters was selected to optimize the complete description of the plasticity with regard to both accuracy and speed of measurement. Several other examples of the measurement of Lepidopteran wing patterns with an image analyzer are given. The advantages and disadvantages of an image analyser are discussed. An image analyser can be an effective tool, especially for complex measurements, but the development of software is critical.

Introduction

The wing patterns of butterflies and moths have been studied in many biological disciplines (for example in genetics (Brakefield 1984, Robinson 1990), developmental biology (Nijhout, 1985, 1991), evolutionary ecology (Endler 1984, Kingsolver 1987). The traditional technique used is to quantify the patterns into classes, as estimated by eye. This may be adequate for discrete morphs but is likely to introduce bias for a more complex variation (see e.g. Brakefield & Dowdeswell 1985). A more detailed quantification of particular pattern elements can sometimes be made using a microscope fitted with a micrometer (e.g. Ehrlich & Mason 1966, Mason et al. 1968, Bowers et al. 1985).

Computers and electronic measuring instruments can provide a more accurate analysis. Examples are a color analyser (Brakefield & Liebert 1985), and a digitizing pad (Strauss 1990, Kingsolver & Wiernasz 1991). An image analyser combines the functions of most electronic measuring instruments. Typically it consists of a TV-camera, a computer and an image processor (often called a frame grabber) that converts the TV-signal into digital form suitable for the computer (Gonzalez & Wintz 1987). Until recently image analysers existed only with mainframe or minicomputers and its use was hardly accessible to entomologists. Daly (1985) concluded that image analysis was not fully developed and of limited use in entomology.

Image analysers which can be built into a personal computer are now readily available to entomologists. Detailed measurements are possible with such a system, not only of distances but also of areas. In addition it also enables analysis of color intensities. Specific parts of an image can be analyzed separately. Simple (e.g. subtractions) or complex (e.g. Fourier analysis for shape) calculations can be performed on the image. Use by entomologists is still very restricted (e.g. Hagerup et al. 1990) but growing. This paper reports on the usefulness of an image analyser for measuring Lepidoptera wings in general.

Anyone interested in using such a system will probably ask two questions:

1) Does it enable the researcher to approach a particular problem in a novel and enlightening way?

2) Does the gain of using an image analyser counter the costs?

We used three criteria to evaluate these questions (in order of importance):

• completeness: A procedure must adequately quantify the pattern with respect to all characters of the wing. Colors or color intensities for example often vary within and between species and in these cases measurements cannot be restricted to sizes.

• repeatability: A procedure is needed which provides a highly repeatable result so that wings can be measured at different times and by different people without influencing the conclusions.

• speed: A procedure is needed which is fast, so that sufficiently large samples can be measured to obtain statistical reliable results.

This paper describes in detail the measurement of wings of *Bicyclus safitza* (Hewitson 1851) with an image analyser, and the difficulties encountered when developing the measurement procedure. With regard to the last criterium (speed) an additional question is addressed: must all characters be measured or can a subset of characters be used to describe the whole pattern of variation? Some other uses of the image analyser in our laboratory are outlined before discussing the general questions asked in the previous paragraph.

MEASUREMENT OF *B. SAFITZA* WINGS WITH THE IMAGE ANALYSER

B. safitza is a satyrine butterfly which occurs throughout tropical Africa (Condamin 1973), and exhibits marked phenotypic plasticity in the form of seasonal polyphenism. The form occurring in the wet season has conspicuous submarginal eyespots and a median white band on its ventral wing surface. The dry season form lacks these pattern elements and is essentially a plain brown butterfly. Intermediates between these extremes can sometimes be found in the field.

Before the image analyser was available to us, forms were evaluated by ranking them into different classes as estimated by the eye, and by measuring the wings with a microscope fitted with a micrometer (Brakefield & Reitsma 1991). With the image analyser we tried to include different measurements (e.g. of color) and more accurate measurements to enable us to estimate the "dry/wetness" of the wings more rigorously. Furthermore, we needed faster measurements for (especially in quantitative genetical analysis) generally large or very large sample sizes are needed to obtain statistically significant results (see e.g. Shaw 1987).

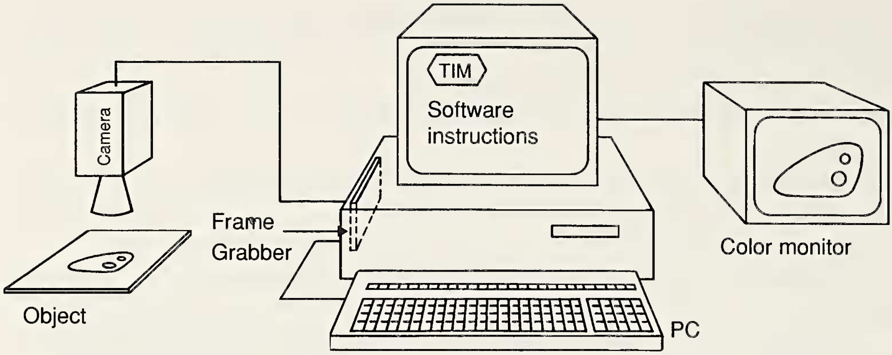


Fig. 1 Set up of the image analyzing system

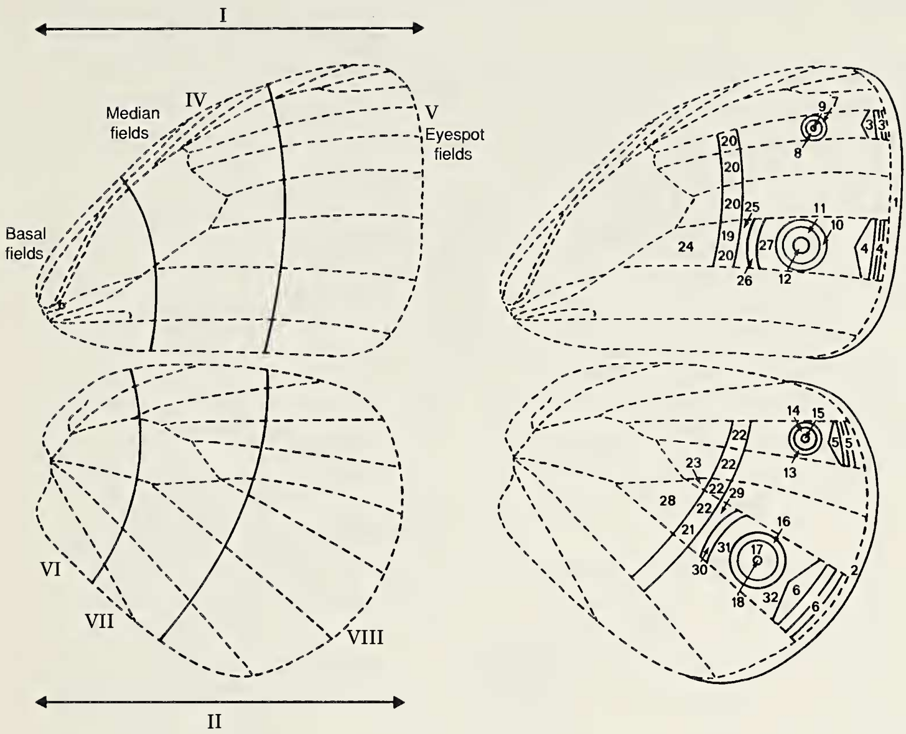
Material and methods.

Measurements. Our image analysis system (fig. 1) was supplied by DIFA Measurement Inc. It consists of a black/white video camera (HTH MO), an IBM-AT compatible Personal computer (Epson PC AX2) with a frame grabber (PC-Vision plus) in one of its additional slots. The video signal is converted by the frame grabber to a digital image. The image is made up of 512x512 pixels, each pixel having a grey value between 0 (=black) and 255 (=white). An additional color monitor displays images; the PC monitor shows the software instructions of an interactive package called TIM (developed by TEA Inc.).

To evaluate the wing pattern of the *Bicyclus* butterflies, a measurement procedure was developed with regard to the three criteria described in the introduction. Software for the measurements was developed partly by DIFA Inc., but for the greater part by ourselves. Results of measurements were written to ASCII files and could easily be used in other (e.g. statistical) programs. Each criterium had its own requirements and the software needed continuous updating. Difficulties and their solutions will be described separately for each criterium.

Completeness. To obtain a range of butterflies from dry to wet season forms, final instar larvae were raised at six different temperatures. The temperatures used were 14°C, 15°C, 17°C, 20°C, 23°C and 28°C, similar to the range of temperatures found in Malawi, the origin of our stocks. Five males and five females were selected at random from the butterflies raised at each temperature. "Dryness" of the butterflies was estimated by eye by ranking them into six classes, from 0 (=dsf, no eyespots and no white band) to 5 (=wsf, large white band and large eyespots). The ranking was done independently by two persons resulting in a nearly identical classification (Spearman rank r= 0.942).

With the image analyser we were able to measure almost any wing pattern character we could think of. A large set containing all potentially important characters of the ventral side was measured for each butterfly (fig. 2). An ANOVA was performed on each of these 67 characters to detect significant differences (at $\alpha = 0.01$) between the six seasonal classes and the sexes. All the areas covaried with wing size and for these an ANCOVA instead of an ANOVA was used. Principal Component Analysis (PCA) was used to provide a more comprehensive picture. Partial correlations with respect to total wing size were used for



Nr.	Object	Measurements:
I,II	total wing	area, contrast area & contrast level
II - VIII	wing fields	area, contrast area & contrast level
1 - 2	fringe	area

Nr.	Object	Measurements:
3 - 6	chevron	area & grey values
7 - 18	eyespots	area & grey values
19 -22	white band	area & grey values
23	white band	distance
24 - 32	white band	grey value (for 32 range & st. dev. of grey values)

Fig. 2 Characters measured for analysis of *B. safitza* wings.

correlations between two area measurements (other than wing area). A mixed matrix of correlations and partial correlations was used for PCA.

Repeatability. A subset of five females was selected to examine repeatability. This sample included two of the most extreme butterflies (1 wsf, 1 dsf) and three intermediates. Characters measured were similar to those of the completeness test. The characters were measured by six different persons, ranging from inexperienced to experienced computer users, and from persons both unfamiliar and familiar with the butterflies. They were all untrained in the specific software program. Coefficients of variation for all six sets of measurements were calculated for each character measured in each wing. The means of these coefficients over all sets of five wings were calculated as an index of repeatability.

Speed. Speed of measurement was evaluated when measuring the wings for the completeness test and for the repeatability test.

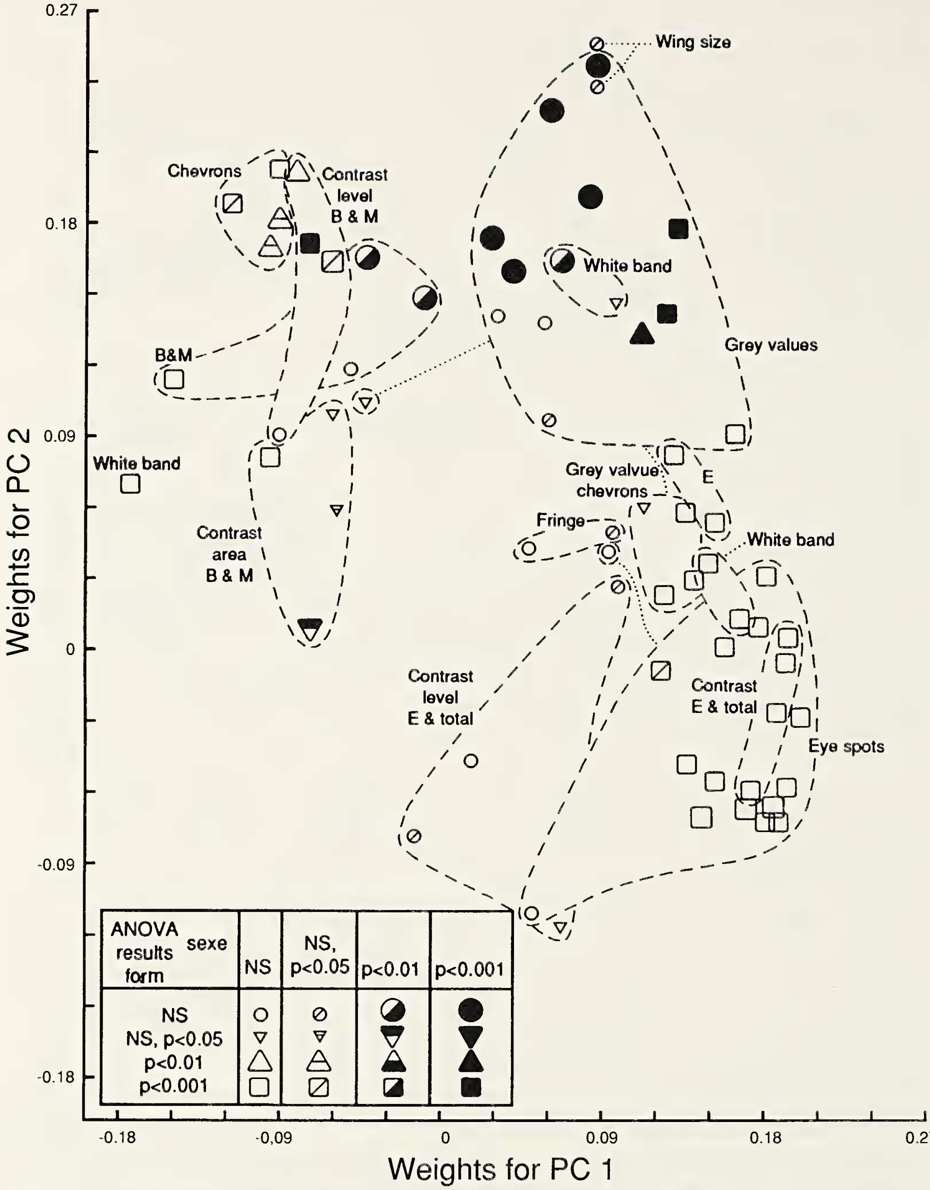


Fig. 3 Weightings for first two principal components in PCA of all characters. Significance of differences in the characters in a multiple ANOVA for sexe and seasonal form are indicated. Names of the groups as in Fig. 2. (E = Eyespot field, M = Median field, B = Basal field).

Results

Completeness. Most characters contribute to the distinction between the seasonal forms and/or the sexes (see AN(C)OVA results in fig. 3). The areas of pattern elements in particular, show significant differences with respect to the seasonal classes in the ANCOVA's (fig. 3, squares and upward pointing triangles). Differences between the sexes are mainly with respect to grey values, or, as in the case of contrast, due to grey values (fig 3, dark symbols).

The PCA ranked the seasonal classes adequately in the first principal component (PC1), the x-axis in fig. 4a. It shows that within each class, as estimated by eye, variation still exists; some butterflies within a class are of a drier/wetter form than others. The sexes are ranked in the second component (PC2), the y-axis in fig. 4a. There is substantial variation within each sexe; some butterflies resemble the opposite sex more than others, especially in the drier forms. To conclude, the measurements of the 67 characters with the image analyser allowed us to make a finer division than ranking them in classes (seasonal or sexual) by eye.

Repeatability. Initially the means of the coefficients of variation ranged up to 28.8%. The high errors were caused by difficulties in separating the different pattern elements. Manual drawing of a line around the pattern element with the mouse was very unreliable. Not everybody was able to draw the lines precisely and different persons often interpreted the elements differently.

Using coloration thresholds (e.g only pixels lower (=darker) than a certain threshold value can belong to the black ring) proved more reliable since a certain threshold always gives the same result for different persons. When each person was allowed to choose their own threshold the different interpretation of pattern elements still caused high errors (COV around 10%). Setting a fixed threshold for each pattern element resulted in acceptable errors (most COV's <2%).

Another source of error was the different exposure to light of the wings when recorded. This influences not only measurements of the intensity of colors, but also all other measurements when a fixed grey value threshold is used for separating the different pattern elements. The internal systems of the image analyser regulating the conversion of different light intensities into different grey values proved very sensitive and vulnerable. So great care always had to be taken to calibrate the system with a standard grey scale, and check for differences with previous measurements.

The most reliable method for separating pattern elements was the use of contrast. Different pattern elements differ in coloration, and consequently differences between neighbouring pixels (= contrast) is highest where two pattern elements border. The software program indicated pixels where contrast was high, and these enclosed elements completely, or left small gaps which could be closed manually. In this way differences between persons are reduced (contrast is always the same) and the influence of exposure is also reduced (contrast is similar at different exposure levels), resulting in COV's of less than 1%.

The white band was very difficult to measure in all forms, regardless of separation procedure, since its outer edge fades into the background and is never clear. Use of a fixed threshold was problematic because grey values within the band varied over the wing. The inner edge however is

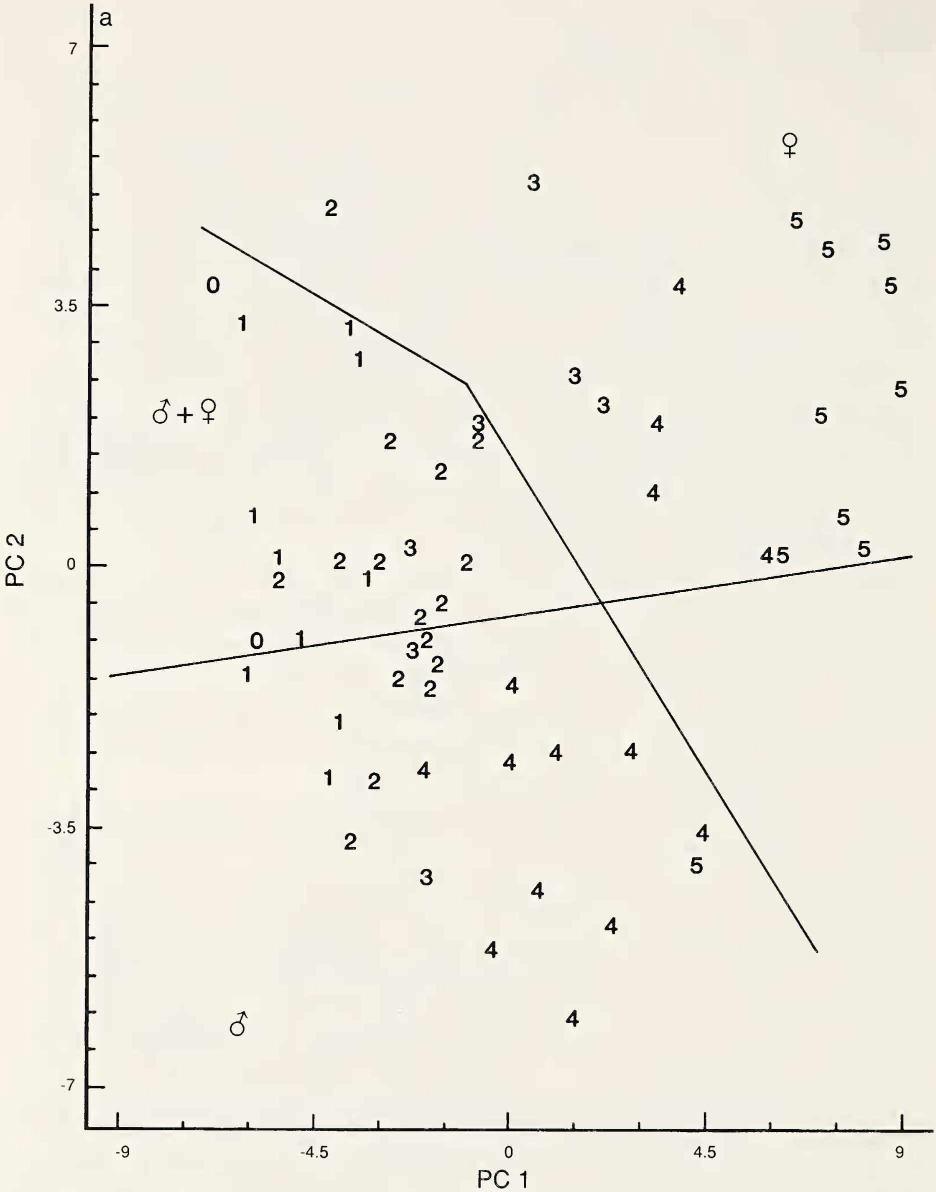
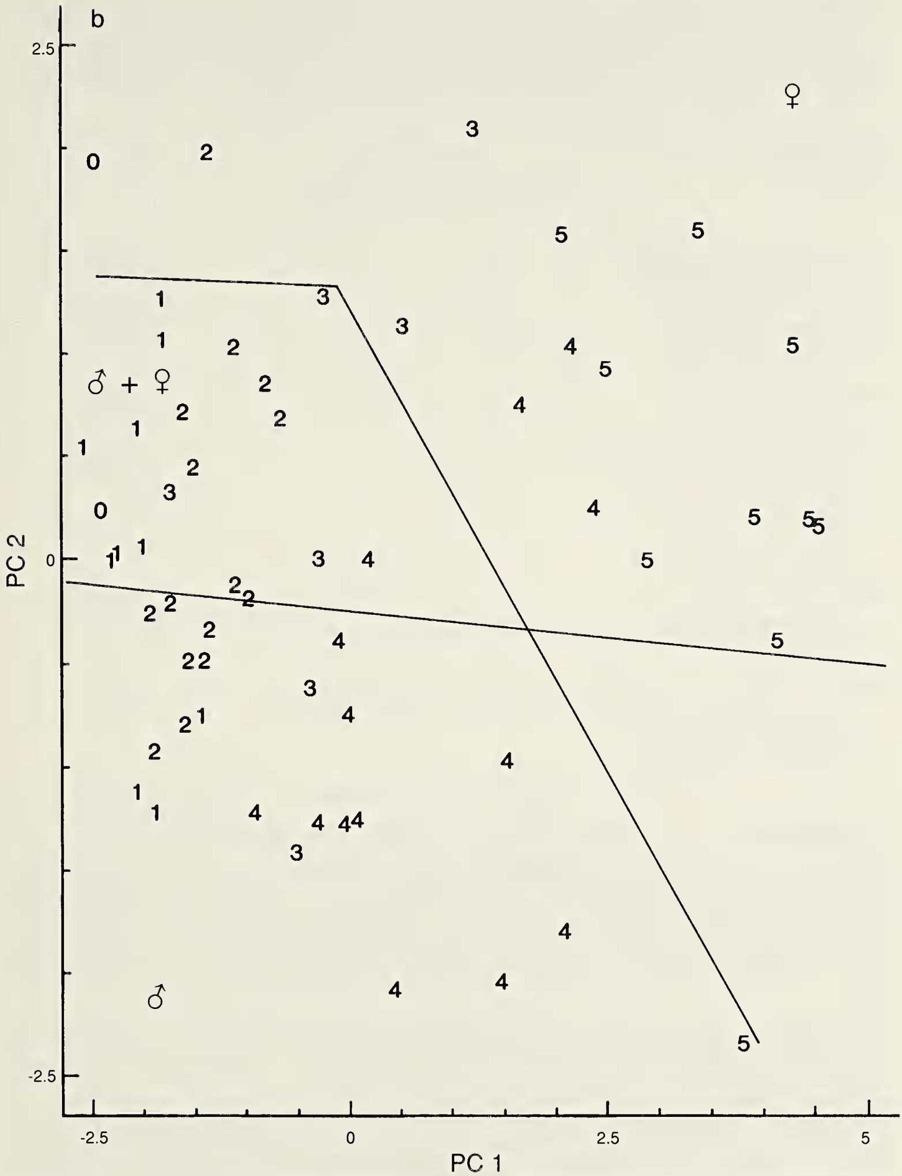


Fig. 4 First two principal components for butterflies in PCA of all characters (a) and for seven selected characters (b, see table 1).

always clear, and it proved to be in a different position in the different forms, according to how well the band was developed. The distance of the white band to the crossing point of two veins proved easy and reliably measurable.

Speed. In theory a software program can be written that finds and measures all pattern elements itself. The only time needed in such a program is for recording and storing the wings; automatic image analysis



can be performed overnight. Such a software program, will, however be very complicated and take a very long time to develop. Instead we used a combined approach, where the greater part of the analysis is automatically performed overnight, and only the functions that are too complicated to program are performed manually.

When contrast was used for separating pattern elements, most time was needed for closing the small gaps in the pixels enclosing the pattern elements. For measuring 7 characters this took together with recording and storing about 7 minutes per wing. When a fixed threshold value was used for separating the pattern elements the only extra time needed was

Table 1. The character subset selected for measurement of B. safitza wings, indicating the amount of variation between seasonal form and sex, and coefficients of variation. Numbers in brackets refer to fig. 2. — = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

Character	AN(C)OVA for season	AN(C)OVA for sex	CV
Area of hind wing (II)	—	*	0.56%
Area of contrast (II)	***	—	1.17%
Area of white pupil in 5th cell (18)	***	—	0.25%
Area of black ring in 5th cell (17)	***	—	0.26%
Distance from crossing point of Cubital & Median veins to edge of band (23)	***	—	0.34%
Mean grey value of midfield in 5th cell (28)	—	***	1.42%
St. dev. of grey values between eyespot and chevron (32)	***	***	0.82%

for indicating where the different pattern elements were located. Together with recording and storing this took less then 1 minute per wing for 7 characters. Because the gain in accuracy of measurement is only small when using contrast instead of fixed threshold values, but the loss in speed is substantial, the fixed threshold method is now always used by us.

Selection of a subset of characters. Characters were selected with reference to the three demands, completeness, repeatability and speed, to reduce the time required to measure one butterfly. Only characters in the 5th cell of the hind wing were selected. This resulted in a small subset of seven characters (Table 1). A PCA comparable to that for all 67 characters was performed on these seven characters. It gave a closely similar result to the PCA for all characters (fig. 4b). The correlation coefficient for both PCA's was 0.92 ($p<<0.001$), showing that there is little loss of information. Each of the major character sets (band, eyespots, color) is represented.

OTHER USES OF THE IMAGE ANALYSER

Crypsis in *Melanitis leda*. A pilot study has attempted to quantify the crypsis of insects at rest by 2-D image analysis of the extent of background matching (see Endler 1984). Six individuals of the polyphenic

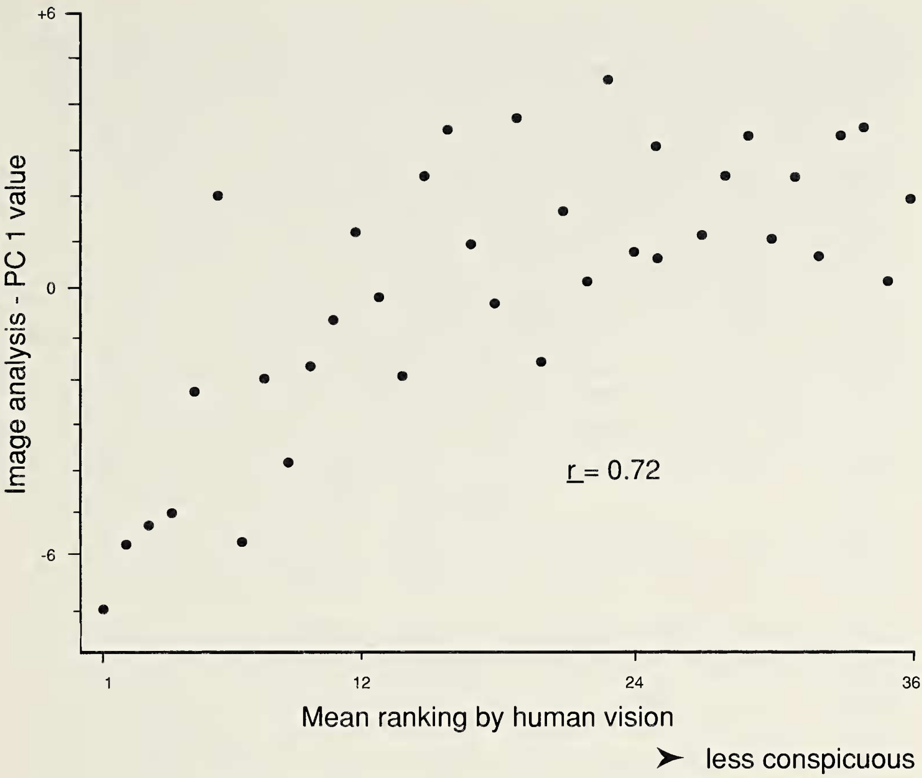


Fig. 5 Crypsis ranking of Melanitis butterflies in photos by the image analyzer vs. the human eye.

satyrine *Melanitis leda*, two wsf and four dsf, were each photographed on a range of six backgrounds; three green leaves and three brown. A comparison was made of the mean grey value, size and shape of the butterfly wings with those of background objects. The same photos were ranked according to conspicuousness of the butterfly by sixteen independent observers. The data were analyzed by PCA. Fig. 5 shows the values of the PC1 for each photo plotted against their ranks. It demonstrates the potential of image analysis for measuring the crypsis of butterfly wings.

***Ephestia kuhniella*.** The measurement of grey values is used to quantify variation in the amount of melanization of the wings within and between different melanic and non melanic genotypes of the flour moth *E. kuhniella* (fig 6). It shows that with the image analyser a finer distinction between genotypes is possible than ranking them as melanic and non melanic.

Development of eyespots in *Bicyclus anynana*. Experiments involving cauterization of cells within developing eyespots in the pupae of *B. anynana* are being performed to investigate the developmental biology of the seasonal polyphenism. Earlier experiments of Nijhout (1981, 1985) indicated that an information gradient is established during pattern determination in the early pupal stage. It is established around

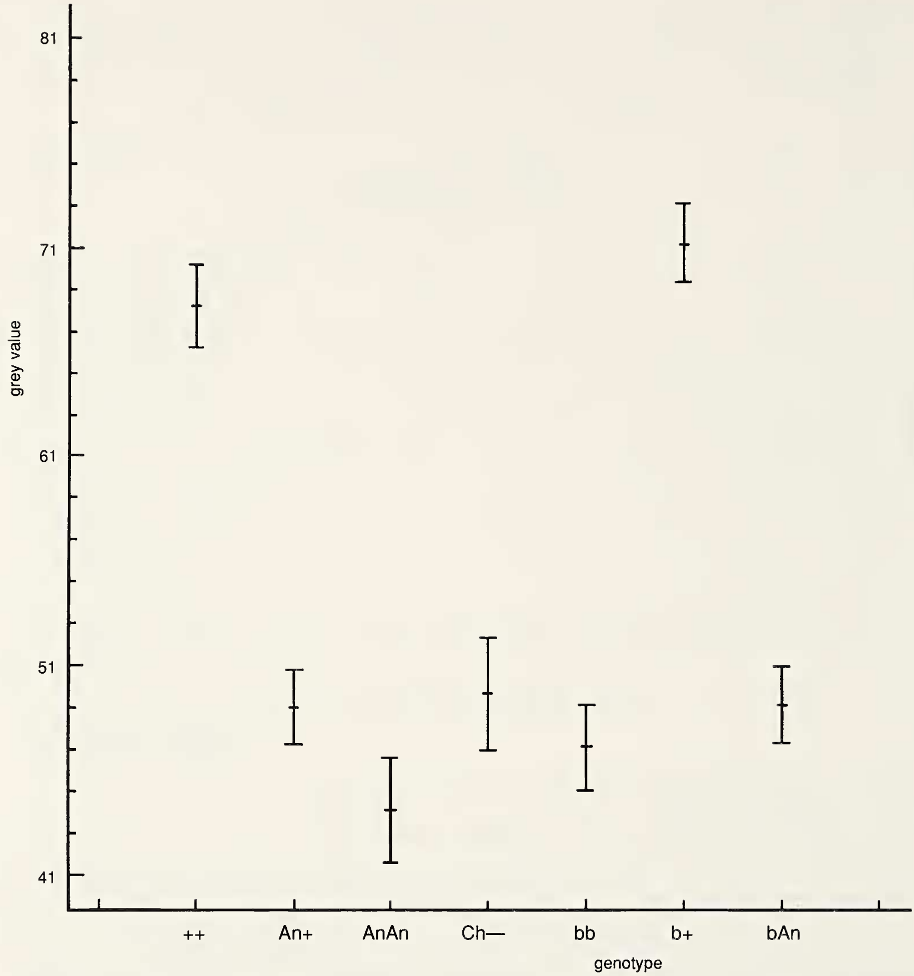


Fig. 6 Mean grey value and 95% confidence intervals for different genotypes of Ephestia moths, as measured by the image analyser. Melanic alleles: An = Alanigra, Ch = Charcoal, b = black (recessive).

a focus of cells within a putative eyespot. Image analysis in combination with the making of Camera Lucida drawings provided the means of accurately quantifying the differences between cauterized and control eyespots at a magnification of about 250x.

Discussion

Biological problems can be approached in a different way (question 1 in the introduction) when analysing lepidopteran wings with an image analyser. The main difference for the analysis of *Bicyclus* wings, is that it is now possible for us to make a better distinction between wing patterns according to seasonal differences. Furthermore, we can process far more wings than is possible with only a microscope. Another advantage is the flexibility of the system as is shown by the other uses of it in our laboratory. It has potential to quantify crypsis, it can distinguish melanic genotypes in *Ephestia*, and it can work at a high magnification.

In general there are several advantages (the gain of question 2, introduction) in using an image analyser for lepidoptera wing measurements. It can perform two types of measurements which most other electronic equipment cannot. It can measure not only distances (like a digitizing pad), but also areas. This can be very useful if for example the total black area in a complicated pattern (e.g. Pierid butterflies, Kingsolver & Wiernasz, 1991) must be evaluated. Furthermore it can measure color intensities, especially in lepidoptera wings often an important character distinguishing between forms, species etc. However continuous variation in color intensity is, though certainly present, rarely studied. This is probably due to the difficulty of measuring it, but with an image analyser this need no longer be the case.

Other advantages are the speed of measurement and the gain in precision of measurements. Both criteria were, however, not easily met and took a lot of software development in our laboratory. If gain in precision of linear measurements is the only goal, it is doubtful whether the purchase of an image analyser will be the best investment. Precision in these measurements can also be increased in other ways (e.g. repeating and averaging). The gain in speed can, however, be considerable. The measurements themselves are faster and the data are directly stored and accessible for computer programs. If analyses are constrained by the time available, purchase of an image analyser might be worth the investment.

There are two disadvantages (the costs of question 2, introduction) of the image analyzer. The first is its costs. Our system, including basic software, cost nearly $20.000. The second disadvantage is its complexity. There are so many functions that can be, and often must be, performed before a measurement takes place, that programs become complex. There is always the possibility of purchasing commercially written programs, but they increase the price and seldom fit the demands of the user completely. It took us a year to modify the basic software, that we purchased commercially, into a program which fully satisfied our demands.

To conclude, the image analyzer can be a valuable tool in analyzing butterfly wings, and probably in other organisms too. It is flexible; both simple and complex analyses are possible. Measurements can be made which are otherwise impossible, and the gain in speed of measurements can be considerable. It is, however, not a cheap system, and one must bear in mind that software development can be a considerable effort.

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